

### Challenge

Miniaturized protein thermal shift assays require fast, precise dispensing and reliable UV detection of limited protein samples.

### Solution

The PULSEspencer R allows rapid, accurate dispensing of small volumes, while the qTOWERiris 384 provides sensitive UV fluorescence detection for high-throughput TSAs.

### Intended audience

Researchers in protein analytics, pharmaceutical development, or labs performing high-throughput stability studies.

## Miniaturized Thermal Shift Assay of $\beta$ -lactoglobulin using PULSEspencer R and qTOWERiris 384

### Introduction

Thermal Shift Assays (TSAs), also referred to as differential scanning fluorimetry, are widely used analytical techniques for assessing the thermal stability of proteins. By monitoring changes in fluorescence as a function of increasing temperature, TSAs provide insights into protein conformational stability under different conditions. TSAs are commonly applied in protein characterization, buffer and formulation optimization, and screening of protein-ligand interactions in both academic research and pharmaceutical development. The miniaturization of such assays is becoming increasingly relevant, as it enables high-throughput screening (HTS) workflows while minimizing sample and reagent consumption. Particularly when working with laboriously extracted or otherwise limited protein samples.

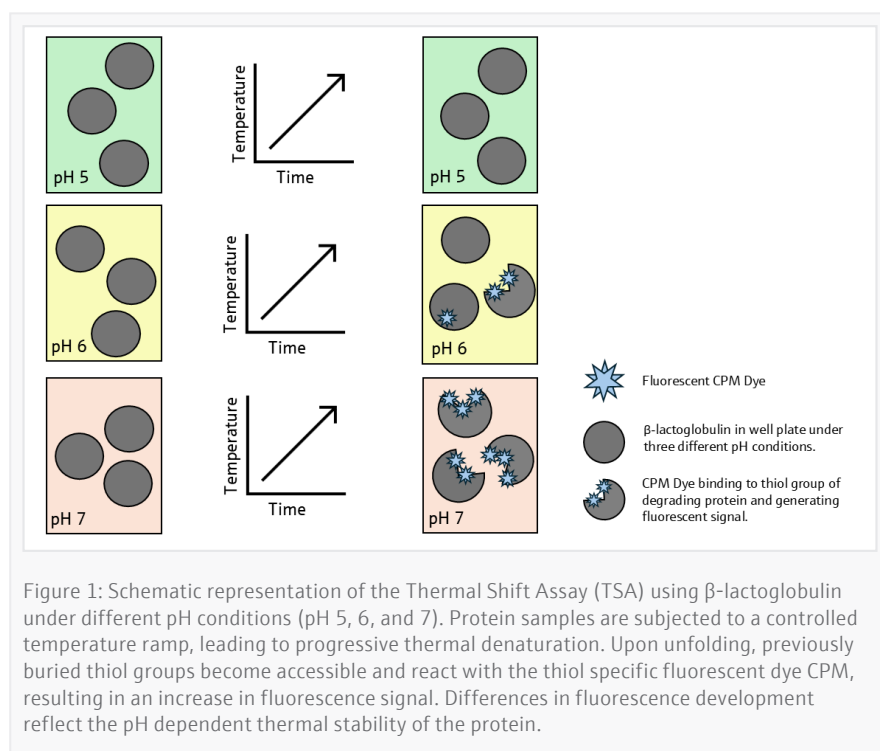
The miniaturization of reaction volumes in a TSA requires fast, accurate, and reproducible dispensing of very small

liquid volumes. The PULSEspencer R is specifically designed to meet these requirements, providing precise and rapid automated non-contact dispensing. This is enabled by digital dispensing based on hp-developed inkjet technology, in which picoliter-sized droplets are generated and precisely combined to achieve target dispensing volumes ranging from the picoliter to microliter scale. By minimizing manual pipetting steps and combining dispensing with automated volume calculation via the Analytik Jena dispenser software, the system substantially reduces assay preparation time and the risk of human error. Consequently, the PULSEspencer R was utilized in this study for TSA miniaturization. To validate its suitability for low volume TSA applications, volumetric performance and dosing reliability of the PULSEspencer R were assessed using the LMX-LUMINA optical volume measurement technology from Liquimetrix.

As a model protein for the TSA,  $\beta$ -lactoglobulin was selected. It is a well-characterized protein of the lipocalin family and exhibits distinct conformational changes in response to variations in pH and temperature. These environmentally triggered structural rearrangements make  $\beta$ -lactoglobulin particularly suitable for studying protein stability and unfolding using TSA-based methods. In this study, its stability was evaluated across three different pH conditions (pH 5, 6, and 7).

For fluorescence signal upon protein unfolding, the thiol-reactive fluorescent dye CPM (7-diethylamino-3-(4-malei-

midophenyl)-4-methylcoumarin) was used. Rapid and reproducible dispensing of small volumes is essential when using fluorescent dyes such as CPM, which are sensitive to light exposure and may degrade during prolonged handling. CPM exhibits minimal fluorescence in aqueous solution but undergoes a strong increase in fluorescence intensity upon covalent binding to free thiol (-SH) groups. During thermal denaturation, buried cysteine residues become exposed as the protein unfolds, enabling CPM binding and resulting in a fluorescence signal (Figure 1).



The temperature at which the rate of fluorescence increase is maximal corresponds to the melting temperature ( $T_m$ ) of the protein. Furthermore, CPM requires excitation in the ultraviolet (UV) range, necessitating detection systems equipped with appropriate optical modules. The qTOWERiris 384 can be equipped with such an optical UV-module and has sensitive detection capabilities, making it ideal for this TSA. Due to the miniaturized assay format, high detection sensitivity is essential to reliably capture the small amounts of dye present in low reaction volumes.

The aim of this study is therefore to evaluate whether a reliable and reproducible CPM-based TSA of  $\beta$ -lactoglobulin can be miniaturized using the PULSEspencer R for fast and accurate liquid dispensing and the qTOWERiris 384 for fluorescence detection. In addition, the LMX-LUMINA system is employed to further validate the dispensing capabilities of the PULSEspencer R.

## Materials and methods

### Reagents and consumables

- ddH<sub>2</sub>O
- NaCl
- PBS (Phosphate buffered saline)
- CPM Dye (7-Diethylamino-3-(4'-Maleimidylphenyl)-4-Methylcoumarin) (257370-100MG, Sigma-Aldrich)
- HEPES (20 mM) (H4034-500G, Sigma-Aldrich)
- $\beta$ -lactoglobulin (L3908-250MG, Sigma-Aldrich)
- 1 x RR4 PULSEspencer Cassette (875-300745, Analytik Jena)
- 1 x RR8 PULSEspencer Cassette (875-300725, Analytik Jena)
- 384 Well PCR Plate, full-skirted, white (844-70039-0, Analytik Jena)
- CyBio TipTray 1536/8  $\mu$ L N [6] (OL5021-25-511, Analytik Jena)
- 2 x Greiner 1536 F Bottom, Hibase microplates (Cat. No. 782101, Greiner)

### Hardware

- PULSEspencer R (875-300010, Analytik Jena)
- qTOWERiris 384, UV-ready, 230V, incl. color module 1 (844-00859-2, Analytik Jena)
- Color Module 7 for qTOWERiris series (844-00826-0, Analytik Jena)
- CyBio Well vario pipettor (frontside, linear transport system) (OL3381-25-300, Analytik Jena)
- CyBio Well vario 1536/8  $\mu$ L pipetting head (OL5021-24-135, Analytik Jena)
- LMX-LUMINA System (Liquimetrix)

### Sample preparation for dispensing

First,  $\beta$ -lactoglobulin was prepared in HEPES buffer at a concentration of 10 mg/mL. Subsequently, PBS buffers were prepared at three different pH values (5, 6, and 7). The CPM dye was initially dissolved in DMSO to a concentration of 20 mM. This stock solution was then diluted 1:40 with buffer and NaCl (2.5  $\mu$ L CPM stock solution, 48.75  $\mu$ L HEPES [20 mM], 48.75  $\mu$ L NaCl [2 mM]) to a concentration of 500  $\mu$ M (Table 1). To ensure optimal performance, the final step of CPM preparation was carried out immediately before dispensing the reagents.

Table 1: Prepared reagents for Thermal Shift Assay (TSA) of  $\beta$ -lactoglobulin at three different pH values 5, 6, and 7.

| Reagent                         | Concentration | Volume        |
|---------------------------------|---------------|---------------|
| $\beta$ -lactoglobulin in HEPES | 10 mg/mL      | 100 $\mu$ L   |
| PBS (pH 5)                      |               |               |
| PBS (pH 6)                      | 1 x PBS       | 1 mL          |
| PBS (pH 7)                      |               |               |
| <b>CPM-Solution</b>             | 500 $\mu$ M   | 100 $\mu$ L   |
| 2.5 $\mu$ L CPM in DMSO         | 20 mM         | 2.5 $\mu$ L   |
| 48.75 $\mu$ L HEPES             | 20 mM         | 48.75 $\mu$ L |
| 48.75 $\mu$ L NaCl              | 2 mM          | 48.75 $\mu$ L |

### Dispensing on PULSEspencer R

For the dispensing of all reagents with the PULSEspencer R, a protocol was created on the Analytik Jena dispenser software. In the protocol, the five reagents were included in the fluid list, all specified as *aqueous solutions without surfactant*, except for the  $\beta$ -lactoglobulin solution, which was specified as *Protein (surfactant-free)*. In this TSA, six different conditions for  $\beta$ -lactoglobulin were investigated. Each condition consisted of a defined pH (pH 5, 6, or 7) and a final reaction volume (5  $\mu$ L or 10  $\mu$ L). For each condition, four wells were assigned in a 384-well PCR plate, resulting in four technical replicates per sample (Figure 2).

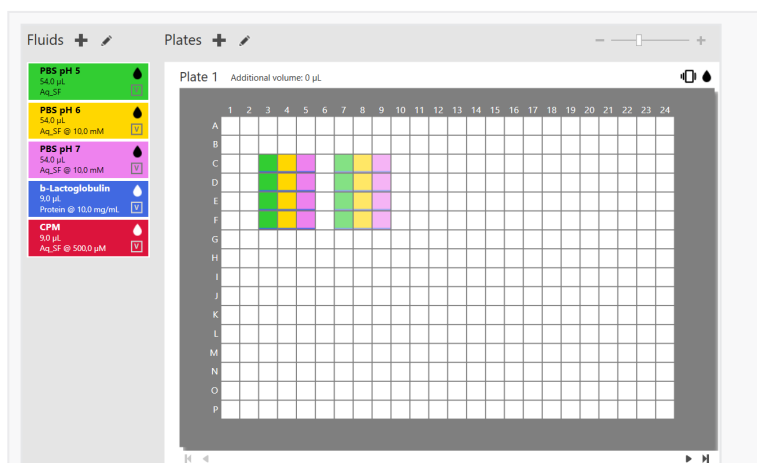


Figure 2: Fluid list (left) and 384-well plate layout (right) in Analytik Jena dispenser software for TSA protocol with  $\beta$ -lactoglobulin on PULSEspencer R. Well columns 3–5 represent final reaction volume of 10  $\mu$ L, well columns 7–9 represent final reaction volume of 5  $\mu$ L. Green columns (3 and 7) represent condition at pH 5, yellow columns (4 and 8) represent pH 6, and pink columns (5 and 9) represent pH 7.

In columns 3–5 of the 384-well PCR plate, the three pH values were analyzed in 4 replicates at a final volume of 10  $\mu$ L per well. The same was applied in columns 7–9 at a final volume of 5  $\mu$ L (Figure 2). Dispensing volumes for each condition were defined using the Set Value function of the Analytik Jena dispenser software, with individual component volumes adjusted according to the specific mixing ratio (Table 2).

Table 2: Dispensed volumes and final concentrations of each component for the two different final volumes of 5  $\mu$ L and 10  $\mu$ L. Dispensed with PULSEspencer R for TSA of  $\beta$ -lactoglobulin.

| Component                               | Volume       | Final concentration | Final volume |
|---|--------------|---------------------|--------------|
| CPM dye solution                        | 0.25 $\mu$ L | 25 $\mu$ M          |              |
| $\beta$ -lactoglobulin solution         | 0.25 $\mu$ L | 0.5 mg/mL           | 5 $\mu$ L    |
| PBS at respective pH value (5, 6, or 7) | 4.5 $\mu$ L  |                     |              |
| CPM dye solution                        | 0.5 $\mu$ L  | 25 $\mu$ M          |              |
| $\beta$ -lactoglobulin solution         | 0.5 $\mu$ L  | 0.5 mg/mL           | 10 $\mu$ L   |
| PBS at respective pH value (5, 6, or 7) | 9 $\mu$ L    |                     |              |

Importantly, for better homogenization of the reagents *Shake during dispense* was turned on in the dispenser software setting. After preparation of this protocol in the software and preparation of all components, the dispensing was performed on the PULSEspencer R. For this, a 384-well PCR plate was inserted into the destination plate holder of the dispenser. The instructions of cassette loading and pipetting of fluids of the Analytik Jena dispenser software were followed and all fluids were dispensed in only 4 minutes and 20 seconds into the respective wells including the manual steps. After the dispensing was completed, the plate was sealed with a PCR sealing foil for subsequent analysis with the qTOWERiris 384.

### Thermal shift assay using qTOWERiris 384

All measurements shown were performed on the qTOWERiris 384 UV equipped with color module 7, which provides the optimal excitation and emission wavelengths for CPM ( $\lambda_{\text{ex}} = 375$  nm,  $\lambda_{\text{em}} = 492$  nm). The protocol in the qPCR software (qPCRsoft version 5) for this application was prepared and executed as follows:

The thermal profile was initiated with an equilibration phase at 5  $^{\circ}$ C for 30 minutes to ensure homogenous sample temperature. Further internal tests suggest that reduced equilibration times may be sufficient for this assay format.

Subsequently, the melting curve was recorded by increasing the temperature from 30 °C to 90 °C. The fluorescence intensity was acquired in 1 °C increments with a 15-second equilibration time at each step (Ramp rate: 2 °C/s).

After the run was finished, the first deviation of the raw data of the fluorescence signal was calculated in qPCRsoft. The data was then exported via csv-file. Mean curves and standard deviations for all 4 replicates per condition were calculated and visualized for result interpreting. The maxima for all mean curves were determined which represent the melting temperatures ( $T_m$ ) for the respective condition.

### Validation of PULSEspencer R dispense volumes with the LMX-LUMINA system

Two Greiner 1536 F-Bottom, Hibase microplates were pre-filled with 4  $\mu$ L of ddH<sub>2</sub>O using the CyBio Well vario equipped with a 1536/8  $\mu$ L pipetting head and CyBio TipTray 1536/8  $\mu$ L N. These plates were then measured using the LMX-LUMINA to establish a baseline. A defined volume pattern consisting of eight target volumes ranging from 50 nL to 600 nL was dispensed using the PULSEspencer R. Each target volume was distributed into 52 wells of a 1536-well plate, resulting in a total of 416 addressed wells. An overview of the dispensed volumes and experimental layout is provided in Table 3.

Table 3: Dispensed volumes and experimental layout used for optical volume verification with LMX-LUMINA system.

| Target volume (nL) | Number of wells | Addressed columns in 1536 well plate |
|--------------------|-----------------|--------------------------------------|
| 600                | 52              | 4–5                                  |
| 500                | 52              | 6–7                                  |
| 400                | 52              | 8–9                                  |
| 300                | 52              | 10–11                                |
| 200                | 52              | 12–13                                |
| 150                | 52              | 14–15                                |
| 100                | 52              | 16–17                                |
| 50                 | 52              | 18–19                                |

A second measurement was performed using the *Compare mode* to quantify the dispensed volume via differential measurement. The results were obtained from the LMX-LUMINA software (Figure 3). An evaporation loss of approximately 20 nL was detected during the dispensing process and considered.

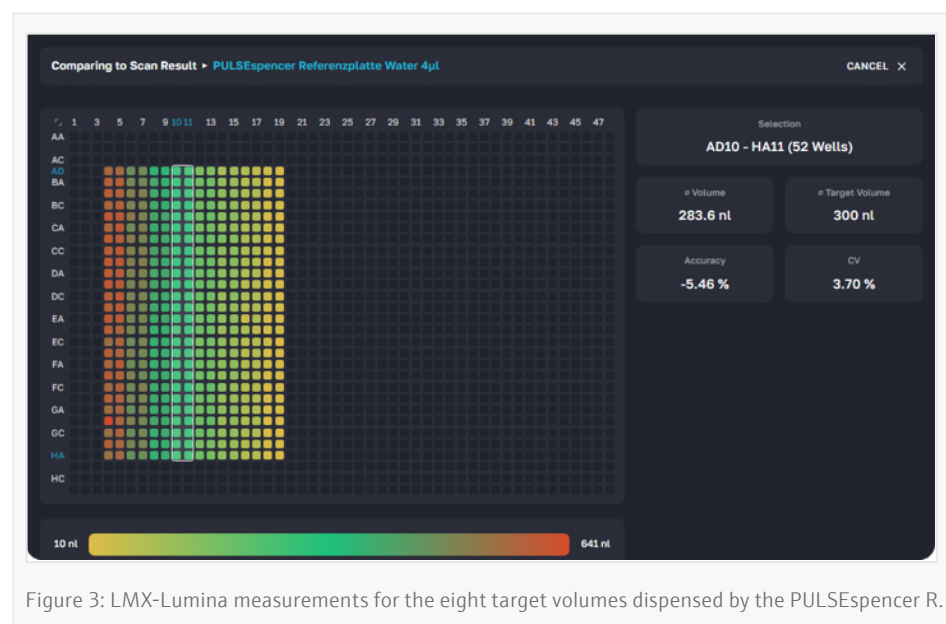


Figure 3: LMX-Lumina measurements for the eight target volumes dispensed by the PULSEspencer R.

## Results and discussion

### Thermal shift assay outcomes

To facilitate comparison across the six experimental conditions (three pH values and two reaction volumes), the fluorescence signals of the four technical replicates were averaged to generate a representative mean melting curve for each condition. The variability within replicates is shown as a shaded standard-deviation envelope around each mean curve, illustrating the consistency of the measurements and allowing direct visual assessment of differences between conditions (Figure 4).

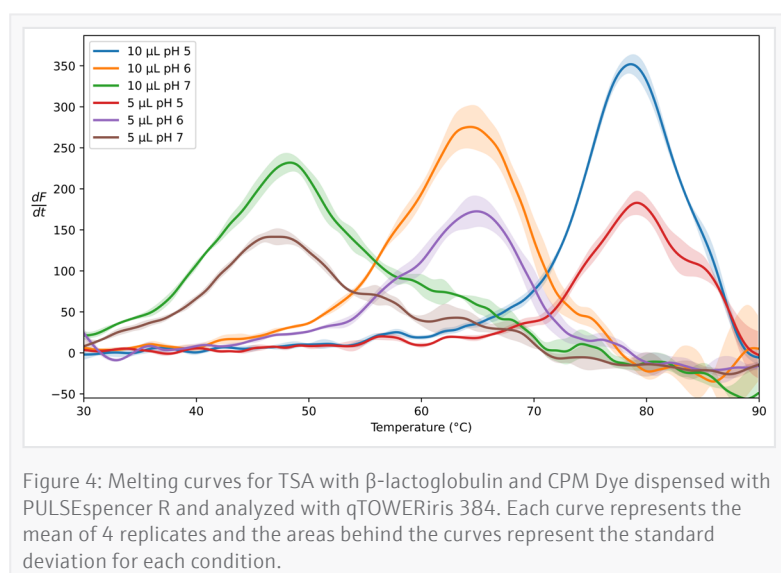


Table 4: Melting Temperature  $T_m$  for each condition determined by the maximum values of the mean melting curves LMX-LUMINA system.

| pH | Volume [ $\mu$ L] | $T_m$ [ $^{\circ}$ C] | SD [ $^{\circ}$ C] |
|----|-------------------|-----------------------|--------------------|
| 5  | 5                 | 79.1                  | 0.06               |
|    | 10                | 78.6                  | 0.93               |
| 6  | 5                 | 64.9                  | 0.33               |
|    | 10                | 64.3                  | 0.71               |
| 7  | 5                 | 46.7                  | 0.67               |
|    | 10                | 48.3                  | 0.42               |

The highest melting temperatures were detected at pH 5, with  $T_m$  values of 78.6  $^{\circ}$ C for the 10  $\mu$ L samples and 79.1  $^{\circ}$ C for the 5  $\mu$ L samples. At pH 6, the thermal stability of  $\beta$ -lactoglobulin was markedly reduced, yielding  $T_m$  values of 64.3  $^{\circ}$ C (10  $\mu$ L) and 64.9  $^{\circ}$ C (5  $\mu$ L). An even further decrease in thermal stability was observed at pH 7, where melting temperatures of 48.3  $^{\circ}$ C for the 10  $\mu$ L samples and 46.7  $^{\circ}$ C for the 5  $\mu$ L samples were determined (Table 4). These results clearly indicate that  $\beta$ -lactoglobulin exhibits its highest thermal stability under acidic conditions, with pH 5 representing the most favorable condition among those tested. This behavior is consistent with previously reported pH dependent conformational changes of  $\beta$ -lactoglobulin, which is known to be more stable under mildly acidic conditions.

Importantly, the melting temperatures obtained from the smaller assay format (5  $\mu$ L final volume) deviated only marginally from those measured in the 10  $\mu$ L format (Table 4). Additionally, the melting curves of the two reaction volumes at identical pH conditions displayed similar shapes and peak positions, differing primarily in peak intensity (Figure 4). This demonstrates that assay miniaturization down to 5  $\mu$ L reaction volumes using the PULSEspencer R is both reliable and reproducible. The ability to accurately dispense very small volumes is particularly advantageous when working with valuable or scarce protein samples, such as purified  $\beta$ -lactoglobulin. Furthermore, the rapid dispensing process of the PULSEspencer R is critical for CPM-based assays, as the dye is sensitive to UV light and can degrade during prolonged handling times. The entire dispensing process for the 24 wells, including manual steps such as loading the plate and cassettes and adding fluids, took just over four minutes. This is over twice as fast as manual pipetting. The miniaturization and speed of the PULSEspencer R highlight its ability to process a high number of samples with minimal consumption.

Finally, the qTOWERiris 384 proved to be highly suitable for CPM-based TSAs. The integrated UV color module provided optimal excitation and emission conditions for CPM, enabling sensitive and reproducible fluorescence detection across all tested conditions. This demonstrates the qTOWERiris 384's versatility, as it is suited not only to qPCR, but also to other applications such as this TSA.

### Validation of PULSEspencer R

To ensure that the reliable TSA results obtained at miniaturized reaction volumes are not limited by dispensing variability, the volumetric performance of the PULSEspencer R was independently verified using the LMX-LUMINA optical volume measurement system. The volumetric verification confirmed the high dispensing reliability of the PULSEspencer R across the tested volume range (Figure 5).

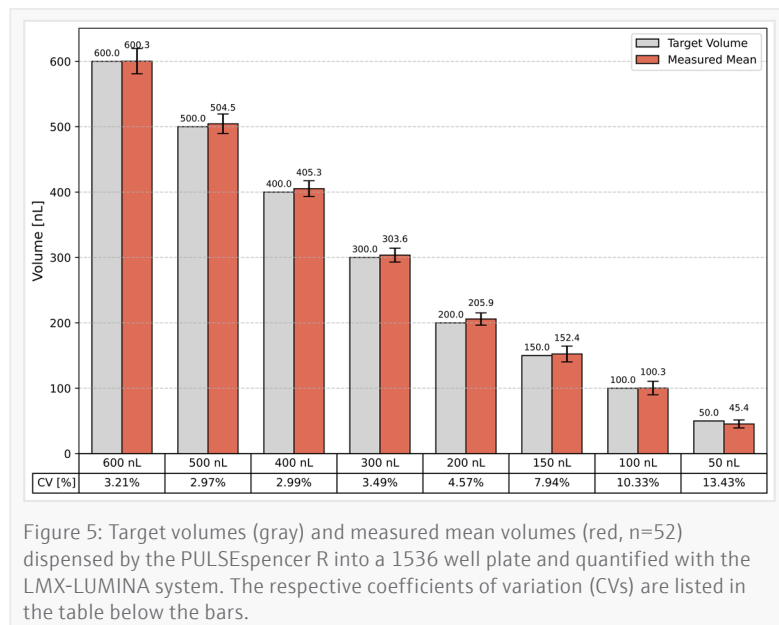


Figure 5: Target volumes (gray) and measured mean volumes (red, n=52) dispensed by the PULSEspencer R into a 1536 well plate and quantified with the LMX-LUMINA system. The respective coefficients of variation (CVs) are listed in the table below the bars.

Across the volume range relevant for the miniaturized TSA (down to 250 nL), the PULSEspencer R demonstrated high dispensing precision with coefficients of variation between 3% and 5%. This confirms that the low-volume dosing step used for assay miniaturization does not introduce significant variability and therefore does not compromise TSA data quality. As expected for optical verification at very low liquid volumes, the CV increased at the lower detection limit of the assay (50 nL). This rise in variability can be attributed to physical and technical scaling effects, including evaporation, surface tension, driven droplet inconsistencies, and increased relative influence of instrument noise at nanoliter-scale volumes.

Notably, although the PULSEspencer R can dispense volumes down to the picoliter range, the LMX-LUMINA system's optical volume verification reaches its practical detection limit at very low nanoliter volumes. Consequently, this optical method cannot quantitatively assess dispensing performance in the picoliter range and was therefore not evaluated in this study.

Importantly, unlike multichannel pipetting, where all wells of a column or row are filled simultaneously, the PULSEspencer R dispenses each well sequentially (single dispense, row-wise). Despite this inherently more demanding dispensing mode, the system still achieved low CVs across the tested volume range. This highlights the robustness of the dispensing mechanism and underlines that the observed precision is not compromised by the sequential actuation principle.

Overall, the data clearly demonstrates that the setup reliably supports assay miniaturization into the low-nanoliter range, confirming its suitability for high-throughput TSA workflows. As a contact-free and non-destructive optical method, LMX-LUMINA offers the potential to obtain process relevant volume information directly during dispensing on assay plates in future workflows, without affecting the samples.

## Summary

The combination of the PULSEspencer R and the qTOWERiris 384 offers a highly efficient and reliable workflow for CPM based TSAs. Enabled by hp developed inkjet based digital dispensing technology, the PULSEspencer R provides rapid, precise, and reproducible non-contact dispensing by generating picoliter sized droplets that are accurately combined to achieve defined target volumes. This enables reliable dispensing and simultaneous mixing of very small volumes, which is essential for assay miniaturization and particularly advantageous when working with scarce or precious protein samples.

Importantly, the system can dispense both surfactant containing and surfactant-free fluids with high precision. In this study, accurate dispensing of surfactant-free protein and dye solutions was successfully demonstrated. This performance is further supported by independent optical validation using the LMX-LUMINA system. The qTOWERiris 384, equipped with a UV color module, provides optimal excitation and emission settings for CPM detection, enabling sensitive and robust fluorescence monitoring.



Figure 6: qTOWERiris 384 (left) and PULSEspencer R (right)

Together, these instruments support high-throughput TSA applications, minimize sample consumption, and maintain assay reliability, demonstrating their suitability for thermal stability studies of proteins such as  $\beta$ -lactoglobulin.

### Recommended device configuration

Table 5: Overview of devices, accessories, and consumables.

| Article  | Article number | Description   |
|--|----------------|---|
| PULSEspencer R   | 875-300010     | PULSEspencer R brings pL-level dispensing to your lab – automating workflows with unmatched accuracy            |
| RR4 PULSEspencer Cassettes                                 | 875-300745     | Dispensing of surfactant-free reagents, 4 dispenseheads, 1nL – 200 $\mu$ L                                      |
| RR8 PULSEspencer Cassettes                                 | 875-300725     | Dispensing of surfactant-free reagents, 8 dispenseheads, 8 pL – 20 $\mu$ L                                      |
| qTOWERiris 384, UV ready                                   | 844-00859-2    | Real-time PCR thermal cycler, 230V, incl. color module 1  |
| Color Module 7 for qTOWERiris                              | 844-00826-0    | Color module for the excitation and emission of fluorescence dyes such as ATTO390, CPM, 1,8-ANS.                |
| CyBio Well vario pipettor (frontside)                      | OL3381-25-300  | CyBio Well vario Pipettor (frontside, linear transport system) base station for head replacement from the front |
| CyBio Well vario 1536/8 $\mu$ L pipetting head (frontside) | OL5021-24-130  | 1536-channel head for parallel transfers of 100 nL to 8 $\mu$ L   |

### Acknowledgement

The volumetric validation of the PULSEspencer R was performed in collaboration with Liquimetrix GmbH, using their LMX-LUMINA system. For further information go to [www.liquimetrix.com](http://www.liquimetrix.com).

**LIQUIMETRIX**

Trademark Notice: The brand names of the third-party products specified in the application protocol are usually registered trademarks of the respective companies or organizations. This document is true and correct at the time of publication; the information within is subject to change. Methods were developed and tested using the following software versions: Analytik Jena dispenser software 4.1.2, qPCRsoft version 5.

#### Headquarters

Analytik Jena GmbH+Co. KG Phone +49 3641 77 70 info@analytik-jena.com  
 Konrad-Zuse-Strasse 1 Fax +49 3641 77 9279 www.analytik-jena.com  
 07745 Jena · Germany

Version 1.0 · Author: FrHu, ChKI  
 en · 03/2026

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